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Temperature and other environmental effects on ammocoete organs in culture

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INTRODUCTION

The optimal temperature and the range suitable for culturing amphibian tadpole hearts *in vitro* have been established (Stephenson, 1967). In order that comparison with the larval stage of a phylogenetically distinct poikilothermic vertebrate might be made, whole hearts and chopped explants of other organs of ammocoete larvae of *Mordacia mordax* (Richardson) were cultured at temperatures ranging from 37 to 5 °C. As one of us (I.C.P) is also carrying out field investigations relating to *Mordacia*, we hoped to discover whether the temperature range suitable for cultures *in vitro* could be correlated with the range occurring in the animal's natural environment. We also hoped to add to the very sparse information available regarding media requirements of cyclostome tissues in culture.

MATERIALS AND METHODS

In Australia, *Mordacia mordax* has a range which extends from Tasmania in the south to a latitude of approximately 34° S. on the east coast (Strahan, 1960). The ammocoetes used in the following experiments, except those in series E (Table 1), were collected at irregular intervals throughout a period of more than a year from a specific site in the Moruya river (N.S.W. east coast, approximately 36° S.). In series E only, ammocoetes of the same species collected from Tasmania (43° S.) were used for comparison. In all cases, collecting was carried out with the aid of an electric fish-shocker.

The larval stage of *Mordacia mordax* occupies several years and at metamorphosis the average body length is approximately 120 mm (Potter, in preparation). First- and second-year larvae provided hearts of suitable size for whole organ cultures. The ammocoetes used in series A–D (Table 1) were in their first year of larval life, while those in series E–G (Table 1) included both first- and second-year animals. The numbers of ammocoetes available for culture were limited, partly because of the relatively small total of suitable animals obtainable at the

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collecting site at any one time, and partly because of the need to conserve a representative field population for current ecological studies.

Cultures were usually prepared within 2–3 days after collection of the ammocoetes. Until required, the animals were maintained in an aerated tank and provided with a suitable substrate. Immediately before dissection they were

Table 1. *Identification of experimental series and temperatures*

Series	Date of culture	Mean body length (mm)	Mean heart rate per min *in vivo*	Organs or tissue explants cultured	Mean room temperature during experimental periods (°C)	Incubation temperatures used (°C)
A	June (1965)	44	50	Whole hearts; chopped liver explants	19	37, 30, 25 20, 15, 5
B	July–Aug.	45	41	Whole hearts; chopped liver explants	18	37, 30, 25 20, 15, 5
C	November	53	50	Whole hearts	23	30, 25, 20 15, 5
D_1 D_2	December	53	48	Whole hearts; chopped liver explants	25	25, 20
E_1 E_2	March (1966), (Tasmania)	86	38	Whole hearts	25	25, 20
F_1 F_2	July	69	52	Whole hearts; chopped liver explants; chopped kidney explants	19	25, 20
G_1 G_2 G_3	August	90 (3 only)	Not rec.	Chopped liver explants; chopped kidney explants; one whole heart in each medium	21	25

immobilized in MS 222 while records of body lengths and heart beat rates were obtained (Table 1). The procedures of washing the animals in detergent and alcohol and of transferring them to Pannett and Compton's saline containing antibiotics followed the routine used for amphibian tadpoles (Stephenson, 1967). Removal of hearts and other organs required (Table 1) was carried out in saline.

General culture methods followed those used for tadpole hearts, but the liquid medium differed in composition and dilution during successive experiments (Tables 2 and 3). Diluted media were prepared by adding deionized distilled water in the required proportion to complete, full-strength media (Table 2). When

diluted media were used, the concentration of Pannett & Compton's saline used for washing during subculturing was correspondingly reduced.

Plasma clots were used in all experiments. They were usually prepared by mixing equal parts of cockerel plasma and chick embryo extract (50 % in Hanks's BSS) In series B only, ammocoete extract was substituted for avian embryo extract. It was also a component of media 2 and 3 (Table 2).

Table 2 *Composition of media**

Medium 1		Medium 2		Medium 3	
Medium 199	7 parts	Medium 199	7 parts	Medium 199	7 parts
Hanks's BSS	7 parts	Hanks's BSS	7 parts	Hanks's BSS	7 parts
Cockerel serum	4 parts	Lamprey serum (*Mordacia*)	4 parts	Cockerel serum	4 parts
Chick embryo extract (50 % in Hanks's BSS)	1 part	Ammocoete extract (50 % in Hanks's BSS)	1 part	Ammocoete extract (50 % in Hanks's BSS)	1 part
Medium 4		Medium 5		Medium 6	
Eagle's minimum essential medium	9 parts	Medium 199	8 parts	Medium 199	8 parts
Foetal calf serum	1 part	Foetal calf serum	2 parts	Cockerel serum	2 parts

* Penicillin sodium G (40 i.u./ml), streptomycin sulphate (50 μg/ml) and mycostatin (20 μg/ml) were added to all media.

In the preparation of ammocoete extract, several first-year animals were skinned completely and degutted from the posterior border of the pharynx to the anus. They were then chopped as finely as possible with curved scissors. An equal volume of Hanks's BSS was added to the chopped tissue, which was then broken down further in a small homogenizer. After standing for 10 min, the mixture was spun at 3500 rev./min for 15 min. The supernatant was passed through a 0·8 μ Millipore filter.

Incubation temperatures ranging from 37 °C or 30 to 5°C were used only for the first three series (Table 1, A–C). In all later series, cultures were incubated only at 25 and 20 °C, both of which had been found to be favourable for cell outgrowth. As initiation and progression of outgrowth were markedly slower than in the case of tadpole tissues, the culture period was extended to 14 days as a minimum. Washing of cultures and renewal of the liquid medium were carried out every second day. Heart beat rates were recorded daily.

Most cultures were fixed in formol-saline and stained in Mallory's aqueous haematoxylin. Those showing particularly good outgrowth were fixed in absolute methanol and stained by the Jenner-Giemsa technique (see Paul, 1960). Pretreatment with silver nitrate was used occasionally. Photographs were taken with a Zeiss Photomicroscope.

RESULTS

(1) *Viability as shown by maintenance of heart beat*

Hearts were cultured at 37 °C in the first two series only (Tables 1 and 3). It was then obvious that 37 °C was an almost immediately lethal temperature for ammocoete heart tissue. Of the twelve hearts cultured at 37 °C, none survived for more than 12 h and within 4–5 h all exhibited irregularity and weakness of beat.

Hearts from series A, B and C, each series involving a different liquid medium (Tables 2 and 3), were cultured at 30 °C. Survival was best in series C, in which

Table 3. *Outgrowth from heart and kidney cultures in relationship to different temperatures and media*

Series	Incubation temperatures (°C)	No. of heart (h) and kidney (k) explants	No. of explants showing outgrowth	Day on which outgrowth was first noticed	Nature of liquid medium (Ref. Table 2)
A	37	8 (h)	—	—	Medium 1, undiluted
	30	8 (h)	—	—	
	25	8 (h)	1	18	
	20	8 (h)	—	—	
	15	8 (h)	—	—	
	5	8 (h)	—	—	
B	As for A	4 (h) at each temp.	—	—	Medium 2, undiluted
C	30	6 (h)	—	—	Medium 3, diluted 5% for 7 days;
	25	6 (h)	5	8	Medium 3, diluted 10% for 7 days.
	20	6 (h)	5	9	
	15	6 (h)	3	14	
	5*	6 (h)			
	20*	6 (h)	2	11	
D_1	25	5 (h)	—	—	Medium 1, undiluted
	20	5 (h)	—	—	
D_2	25	5 (h)	—	—	Medium 1, diluted 10%
	20	5 (h)	—	—	
E_1	25	5 (h)	2	13	Medium 1, undiluted, 8 days;
	20	5 (h)	—	—	Medium 3, undiluted, days 8–11;
					Medium 3, diluted 10%, days 11–14;
E_2	25	5 (h)	3	9	Medium 1, diluted 10%, 8 days;
	20	5 (h)	1	10	Medium 3, diluted 10%, days 8–14
F_1	25	5 (h), 6 (k)	4 (h), 5 (k)	5	Medium 3, diluted 10%
	20	5 (h), 6 (k)	3 (h), 5 (k)	6	
F_2	25	5 (h), 6 (k)	4 (h), 6 (k)	5	Medium 4, diluted 10%
	20	5 (h), 6 (k)	5 (h), 5 (k)	6	
G_1	25	1 (h), 10 (k)	1 (h), 10 (k)	8 (h), 4 (k)	Medium 5, diluted 10%
G_2	25	1 (h), 10 (k)	1 (h), 10 (k)	6 (h), 4 (k)	Medium 6, diluted 10%
G_3	25	1 (h), 10 (k)	1 (h), 10 (k)	6 (h), 4 (k)	Medium 1, diluted 10%

* Cultures incubated at 5 °C for 7 days, then transferred to 20 °C.

five out of six hearts were beating on day 5 and one on day 6. None of the eighteen hearts in the three series survived until the seventh day.

At 25, 20, 15 and 5 °C, almost total maintenance of beat throughout the entire culture period was the general rule, regardless of the nature or the concentration of the medium. Series B (Table 3) was exceptional in that although viability at 15 and 5 °C was good, most hearts at 25 and 20 °C stopped beating within the first few days. As the environmental conditions for the hearts in this experiment differed from those in all other series (Table 3) and as these conditions were not repeated, the reason for the unusual result is uncertain. Of possible significance is the fact that series B was the only series in which ammocoete extract replaced chick embryo extract in the plasma clots. In most cases the clots with ammocoete extract did not set satisfactorily and tended to liquefy early, especially at higher temperatures.

(2) *Rate of heart beat*

As in the case of tadpole hearts (Stephenson, 1967) the mean rate of heart beat per minute in culture usually remained well below the mean rate recorded *in vivo* (Table 1). Occasionally, an individual heart maintained a very high rate even at a comparatively low temperature, but in general the overall relationship of heart beat rate to temperature was adequately defined.

A complicating factor was the relatively high frequency of temporary or permanent asynchrony of auricular and ventricular beats. This phenonomen, only rarely noted in cultured tadpole hearts, was usually seen in at least one or two hearts in each ammocoete series. It appeared to have no relationship to specific temperatures or to the nature or concentration of the medium. It could start or stop without obvious cause. When asynchrony occurred, the ventricular beat was usually more regular than that of the auricle. Although both beats were recorded separately, the ventricular beat alone was used in the calculation of the mean.

The initial period of adjustment to incubation temperatures appeared to be longer than in the case of tadpole hearts. For the first 2 or 3 days in culture (Text-figs. 1, 2) no constant pattern in relation to different temperatures could be detected.

At 37 °C, the period of viability was so short (section (1) above) that the hearts very quickly showed irregularity of beat and decrease in rate. An initial rise in rate was sometimes noted within the first 2 or 3 h. At 30 °C, a decline in rate and strength of beat began by the second day and continued (Text-figs. 1, 2) until all hearts had stopped beating.

By the fourth day, the highest mean rate in all series involving a full range of incubation temperatures was found at 25 °C (Text-fig. 1). In general, a progressive decrease of rate accompanied successively lower incubation temperatures (Text-fig. 2) but temporary overlaps (Text-fig. 1, day 4) were not uncommon. Transfer of six hearts from 5 to 20 °C was accomplished without loss of viability and with marked increase in rate of beat (Text-fig. 2).

In series in which hearts were cultured only at 25 and 20 °C (Table 3), the rate of heart beat was in general higher at 25 °C. Evidence was too limited to permit any conclusive comparisons of rate of beat in relation to different media.

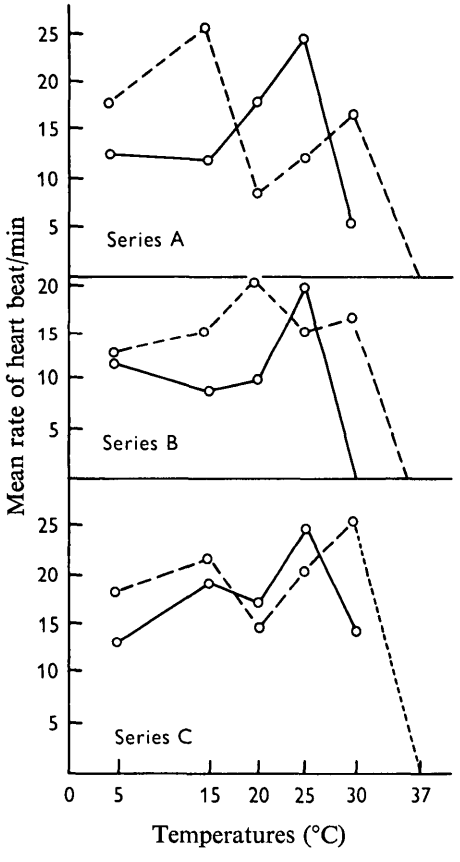

Fig. 1

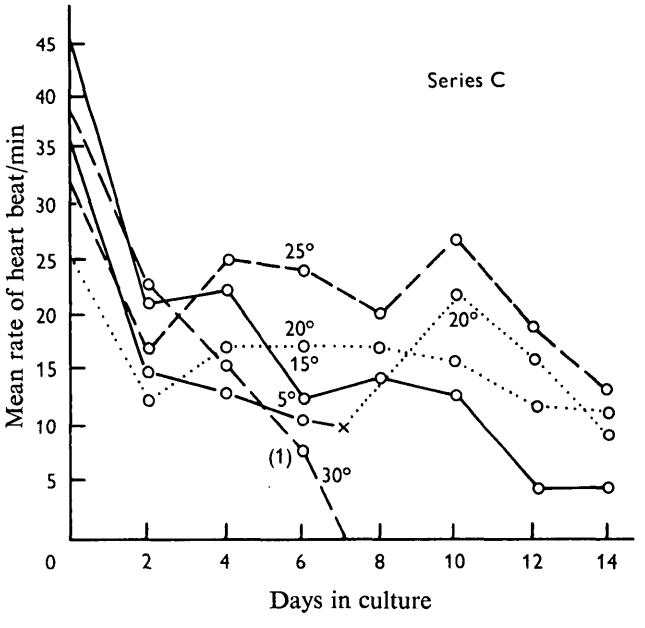

Fig. 2

Text-fig. 1. Comparison of initial reaction of heart beat to incubation temperature (day 1) with rate after period of adjustment (day 4). Day 1 is indicated by broken lines, day 4 by unbroken lines. In series C, actual incubation of hearts at 37 °C did not occur.

Text-fig. 2. Series C. Graphical illustration of the influence of different temperatures on heart beat rate during two weeks in culture. Except at 30 °C., where the figure in brackets indicates the number of hearts still beating, each point indicates the mean rate per minute for six hearts. Hearts originally cultured at 5 °C were transferred after 7 days (point *X*) to 20 °C.

(3) *Extent and type of cell outgrowth*

Outgrowth of cells from heart cultures occurred at 25, 20 °C and, much more slowly, at 15 °C (Table 3). Kidney explants were cultured only at 25 and 20 °C, at both of which temperatures outgrowth occurred. Of the only two successful

cultures of liver obtained (see below), one produced outgrowth at 25 °C and one at 20 °C.

No quantitative measurements of outgrowth were made. Outgrowth of heart cultures almost invariably occurred in irregular patches (Plate 1, fig. A), while other areas of cells lay directly beneath the heart itself (Plate 1, fig. B). Outgrowth from kidney cultures also tended to be irregular. From general observation, however, 25 °C appeared to be optimal with regard to rate of cell migration but reasonably good outgrowths were often obtained at 20 °C. Although comparative estimates of the effectiveness of different media in promoting outgrowth were not possible, the results show that outgrowth of cells from ammocoete organs will occur in a variety of media, provided that these are adequately diluted.

(a) Outgrowth from whole heart cultures

Outgrowth was almost entirely in the form of irregular, epithelioid sheets (Plate 1, figs. A–C). Migration of the cells was relatively slower than that from kidney explants. Even after 14 days, examples of nuclear budding or fragmentation, so frequent in tadpole heart cultures, were seldom found but mitoses were common (Plate 1, fig. D). Anchoring threads or bridges of cells (Stephenson, 1967) were occasionally present; a few small, amoeboid leucocytes were sometimes seen.

Table 3 indicates the number of heart cultures which produced outgrowth and the day on which cells were first seen. For series A, medium 1 (Table 2) which was identical with that used successfully for tadpole heart cultures, was used undiluted. Although the hearts remained viable and continued to beat, no outgrowth was seen until the eighteenth day in culture, when one heart at 25 °C produced a small sheet of cells.

For series B, an undiluted medium was also used (Tables 2 and 3) but the avian components, except for plasma in the clots, were replaced by ammocoete extract and serum from adult specimens of *Mordacia*. No outgrowth was produced and viability at temperatures above 15 °C was poor (section (1) above). As the same medium was not used again, its possible effectiveness in a diluted state is not known.

For all subsequent series (Table 3), the liquid medium was diluted. In series C, outgrowth began only after the medium had reached a dilution of 10 %. It then continued successfully throughout the rest of the culture period.

It was not at first certain whether outgrowth was a result of a dilution factor alone or whether the homologous component present in medium 3 (Tables 2 and 3) was also necessary. For series D, the original avian/synthetic medium (Table 2, medium 1) was used in concentrated and diluted forms at 25 and 20 °C (Table 3). For an unexplained reason no outgrowth occurred in any cultures, a result which suggested that a homologous component might also be necessary. This suggestion was strengthened by the results of series E (Table 3) in which outgrowth was not initiated until the original avian/synthetic medium (medium

1), even in a diluted form, had been replaced by a diluted medium (medium 3) containing ammocoete extract.

Results from the last two series (Table 3, F and G) made it clear, however, that outgrowth was not dependent on any homologous factor. Provided that the dilution was adequate, chemically defined media, such as medium 199 or Eagle's minimum essential medium (Eagle, 1959) enriched with heterologous serum, were effective in promoting cell outgrowth.

(*b*) *Outgrowth from chopped kidney explants*

Kidney tissue provided the highest percentage of successful cultures per series, the fastest rate of migration and the greatest variety of cell types. Kidney explants were cultured at 25 and 20 °C in all types of media used for series F and G (Tables 1 and 3). In series F, over 87 % of twenty-four kidney explants in both media (Table 3) produced outgrowth. In series G, all of the ten cultures in each medium were successful (Table 3) and the outgrowth, though typically irregular, was often extensive (Plate 2, fig. A).

In most cases, blood cells of varying sizes and types began migration within the first 24 h, but epithelioid and fibroblastic migration did not begin until at least the fourth day in culture. Some explants produced very mixed types of outgrowth which, in addition to blood cells, included epithelioid sheets (Plate 2, figs. B, C), fibroblasts (Plate 2, figs. A, D) and occasional pigment cells. The epithelioid cells from kidney had the same widely spaced appearance as those found in tadpole heart cultures (Stephenson, 1967) and ammocoete hearts (Plate 1). When treated with silver nitrate their true boundaries became apparent (Plate 2, fig. C). By the end of a fortnight, some of the epithelioid cells had begun to show slight traces of nuclear budding and fragmentation, while binucleate and even multinucleate cells were not uncommon (Plate 2, fig. C). In one or two cases, early stages of aggregations and fusions of small blood cells to form multinucleate giant cells were noted. The greatest number of nuclei counted in any of these syncytial bodies was thirteen.

Cells at the edges of the epithelioid sheets showed a tendency to become detached (Plate 2, fig. D) and it was often difficult to distinguish what were originally epithelioid cells from fibroblasts. The size of both fibroblasts and epithelioid cells contrasted greatly with the very much smaller blood cells (Plate 2, fig. D). The

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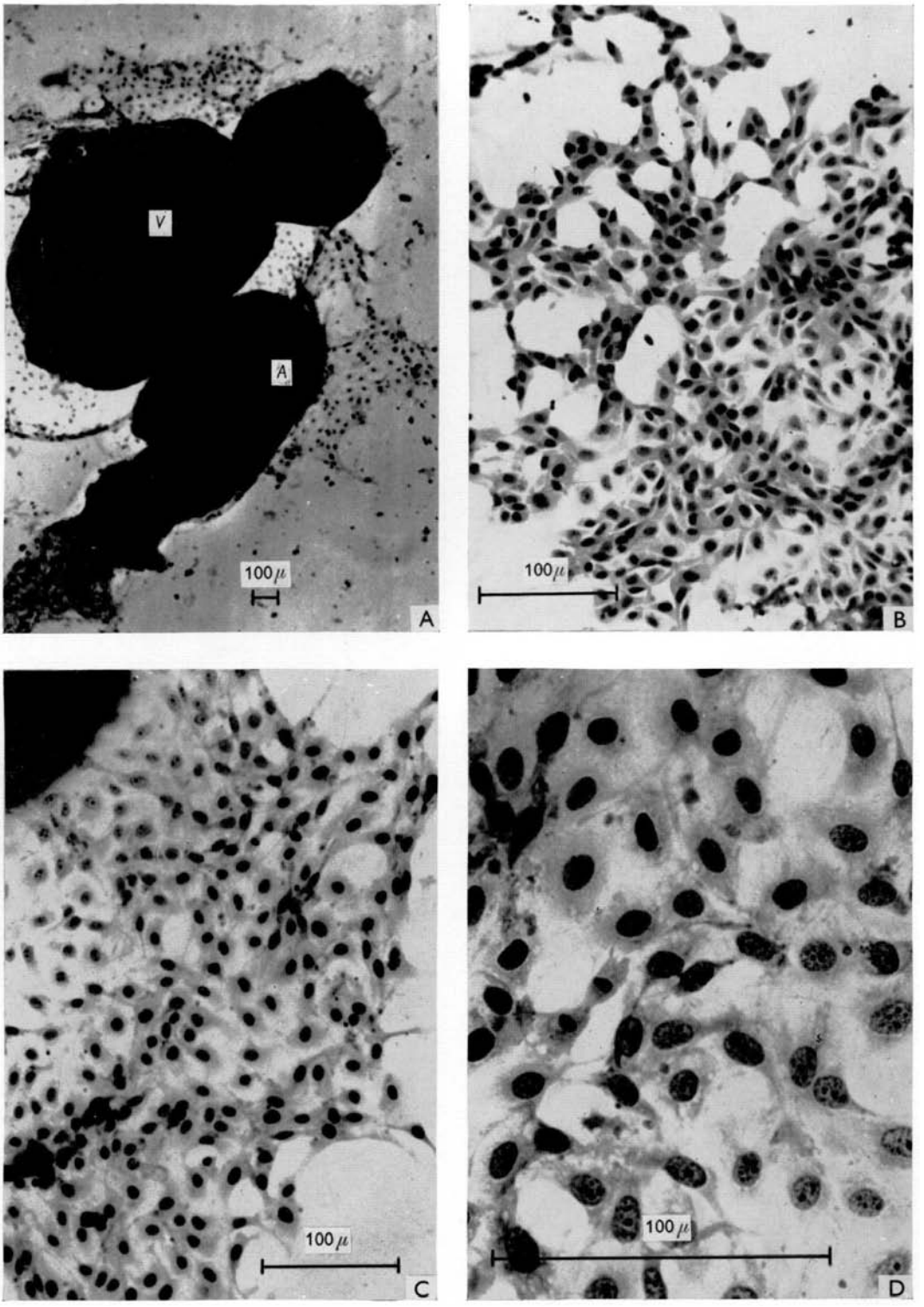
PLATE 1

Fig. A. Whole heart and patches of outgrowth. Series C, 14 days, 20° C. Medium 3, diluted 10 %. *A*, auricle; *V*, ventricle.

Fig. B. Cells originally lying under the heart and exposed after its removal. Series G_1, 14 days, 25° C. Medium 5, diluted 10 %

Fig. C. Epithelioid sheet of outgrowth from heart. Series F_2, 14 days, 20 °C. Medium 4, diluted 10 %.

Fig. D. Detail of part of the outgrowth illustrated in fig. C.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A
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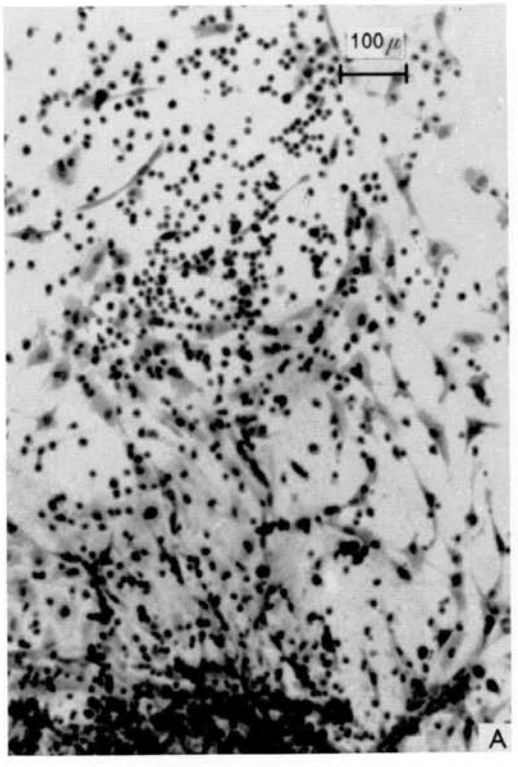
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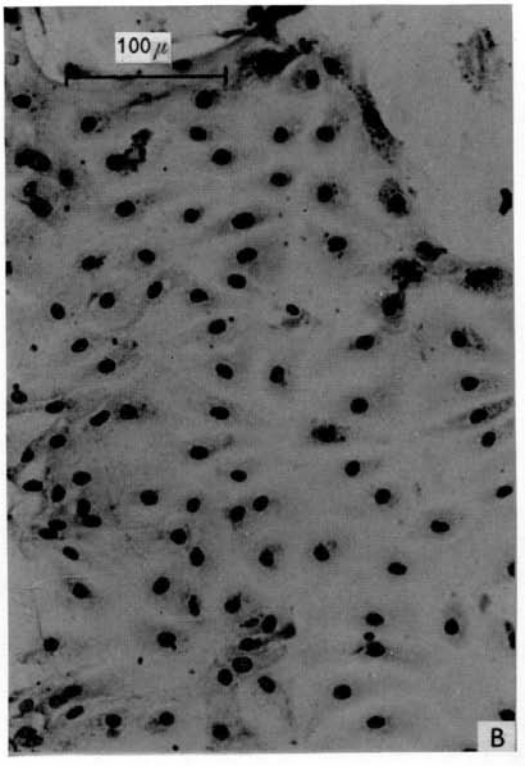
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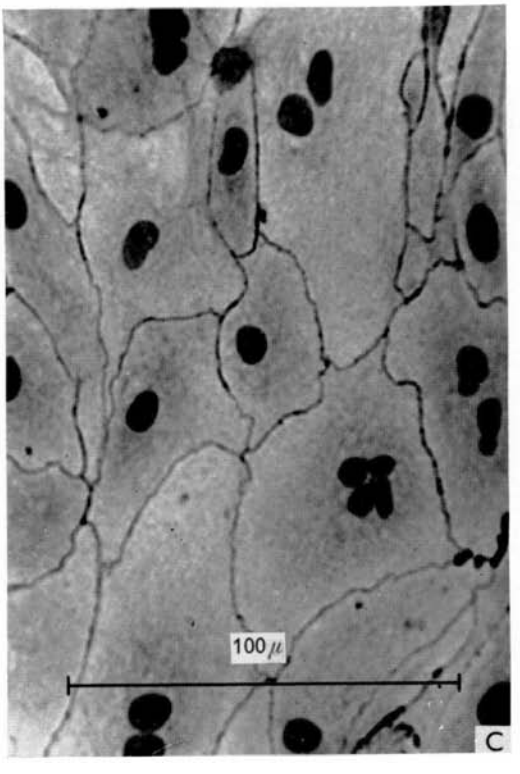
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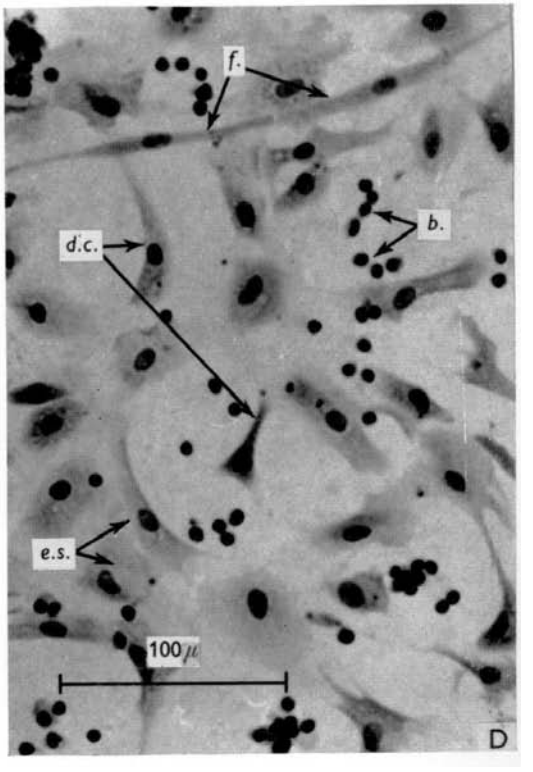
f.
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latter varied slightly in size among themselves and were usually markedly amoeboid. As an ammocoete kidney is typically a site of haemocytopoiesis (Torrey, 1938), the emerging blood cells presumably included haemoblasts or lymphocytes (Jordan & Speidel, 1930) as well as transitional forms.

(*c*) *Outgrowth from chopped liver explants*

Although liver explants were cultured in all media and at all temperatures in which satisfactory outgrowths were produced from heart and kidney cultures (Tables 1, 3), migration of cells from liver tissue was observed in only two cases. The first, in which a few fibroblasts were visible after 2 weeks in culture, was one of eight liver explants incubated at 25 °C in medium 4 diluted 10 % (Table 2). From the second, which was one of eight explants cultured at 20 °C in medium 1 diluted 10 % (Table 2), epithelioid cells began migrating after 6 days. In all other cases, outgrowth was absent from liver cultures and the stained explants usually had a degenerate appearance. In three cultures, part of the bile duct remained attached to a liver explant. Outgrowth was produced from this adhering tissue but not from the liver. The activity of cilia in the bile duct was particularly noticeable.

DISCUSSION

Comparison of results from the present investigation with those relating to amphibian tadpoles (Stephenson, 1967) shows that the lethal and optimal temperatures for ammocoete hearts in culture are lower than those for tadpole hearts. A temperature of 30 °C apparently affects ammocoete hearts *in vitro* in much the same way that 37 °C affects cultured tadpole hearts, i.e. it is ultimately lethal but permits viability to continue for several days. The optimal temperature for rate of beat and for cell outgrowth from ammocoete hearts appears to be 25 °C which, like the 30 °C optimum for tadpole heart cultures, is not far below the corresponding lethal temperature. It is not yet known whether an optimal temperature of 25 °C applies specifically to *Mordacia* tissues *in vitro* or whether it has a wider application to cyclostome cultures generally. The present study has shown no evidence of organ specificity involving temperature preferences.

Growth rate records of ammocoetes from the Moruya site appear to indicate

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PLATE 2

Fig. A. Blood cells, fibroblasts and epithelioid cells in outgrowth from kidney explant. Series G_2, 14 days, 25 °C. Medium 6, diluted 10 %

Fig. B. Sheet of epithelioid outgrowth from kidney. Series F_1, 14 days, 20 °C. Medium 3, diluted 10 %

Fig. C. Epithelioid cells from kidney outgrowth, treated with silver nitrate. Series G_1, 14 days, 25 °C. Medium 5, diluted 10 %.

Fig. D. Cells from kidney outgrowth. Series G_2, 14 days, 25 °C. Medium 6, diluted 10 %. *b*, blood cells; *f*, fibroblasts; *e.s.*, cells of epithelioid sheet; *d.c.*, cells becoming detached from edge of sheet.

that growth is very slight during the winter months of May–August (Potter, in preparation). Yet *in vitro*, when provided with suitable temperatures and media, cells from ammocoetes collected during the winter show no apparent reduction of proliferation and migratory activity. In general, however, outgrowth from ammocoete heart and kidney cultures is relatively much slower than that of corresponding tadpole tissues in culture. It seems possible that the relative rates of cellular activity *in vitro* are expressions of the differences in overall growth rates of ammocoetes and tadpoles under natural conditions.

Monthly records of maximum and minimum water temperatures for over a year at the Moruya collecting site (Potter, in preparation) have shown a maximum summer temperature of 31·5 °C and a minimum winter temperature of 4 °C. Water temperatures of 30 °C and over are, however, exceptional and of short duration. In addition, the substrate in which the ammocoetes are buried probably affords them some protection. Records of water temperatures in Tasmania have not so far been available but it is likely that they are in general considerably lower than at the Moruya site. From the limited evidence provided by the single series of heart cultures from Tasmanian ammocoetes, 25 °C again appeared to be optimal for rates of heart beat and cell outgrowth.

The upper limit of temperatures encountered by ammocoetes of *Mordacia* in their natural habitat seems to correspond reasonably well to the upper limit possible for at least temporary maintanance of explants *in vitro*. If lethal temperatures for isolated organs and tissues in culture bear any direct relationship to lethal temperatures for whole animals in the field, the susceptibility of cultured ammocoete tissues to prolonged exposure at or above 30 °C possibly helps to explain the northern limit of distribution of the species.

Little information relating to the culture of cyclostome organs or tissues appears to be on record. Chlopin (1925) indicated that tissue explants of *Petromyzon fluviatilis* had been cultured successfully at 22–24 °C in diluted rabbit plasma, with or without the addition of lymph extract. Pfeiffer (1935), using Chlopin's medium and an incubation temperature of 16–18 °C, cultured what were claimed to be spleen explants of *Petromyzon*. In view of the absence of the spleen as a discrete organ in lampreys and the morphological relationships of splenic tissue with the typhlosole of the gut (Jordan & Speidel, 1930; Raunich, 1949), the exact identity of the explants is open to question. It is clear, however, that migration of lymphocytes and later of fibroblasts occurred in a heterologous medium. The ability of heretologous media to promote cell outgrowth from cyclostome tissues has been further demonstrated by the present investigations, in which a number of combinations of defined media with heterologous natural media have been used successfully. Dilution of standard media by approximately 10 % appears to be necessary for cell outgrowth from *Mordacia* explants but precise investigations regarding the optimal osmotic concentration have not been made.

SUMMARY

1. Whole hearts of ammocoete larvae of *Mordacia mordax* were cultured at temperatures ranging from 37 to 5 °C.

2. Rapid death of cultures occurred at 37 °C. A temperature of 30 °C was ultimately lethal but viability as indicated by heart beat could be maintained for up to 6 days. Total viability was usually maintained in temperatures from 25 to 5 °C.

3. Rate of heart beat varied in absolute values but the relative pattern in relationship to incubation temperatures was more or less constant. The optimal temperature was 25 °C.

4. Cell outgrowth occurred at 25, 20 and 15 °C. The optimal temperature for rate of outgrowth appeared to be 25 °C, but cultures at 20 °C were also satisfactory.

5. Chopped kidney and liver explants were also cultured. Successful outgrowth was obtained from almost all kidney cultures at 25 and 20 °C. Outgrowth from liver was extremely rare and occurred only at 25 and 20 °C.

6. Successful outgrowth was obtained in media lacking any homologous component. Dilution of standard media by approximately 10 % appeared to be necessary for outgrowth but viability could be maintained in undiluted media.

7. A correlation appears to exist between the upper limit of temperature suitable for maintenance of ammocoete tissues *in vitro* and the highest temperatures encountered by the whole animals in their natural environment.

RÉSUMÉ

Effet de la température et de l'environnement sur les organes de l'ammocète en culture.

1. On cultive des coeurs entiers de larves ammocètes de *Mordacia mordax*, à une température comprise entre 37 et 5 °C.

2. A 37 °C la mort intervient rapidement. Une température de 30 °C est finalement léthale, mais le coeur peut se maintenir en vie pendant 6 jours et poursuivre ses battements. La viabilité est généralement totale à une température de 25 à 5 °C.

3. Le rythme des battements cardiaques varie en valeur absolue, mais sa valeur relative en fonction de la température d'incubation est plus ou moins constante. La température optimale est 25 °C.

4. Une croissance apparaît à 25, 20 et 15 °C. La croissance est meilleure à 25 °C, mais à 20 °C les cultures sont aussi satisfaisantes.

5. On cultive également des fragments de rein et de foie. Une croissance a été obtenue à partir de presque tous les explants de rein à 25 et 20 °C. Pour ce qui concerne le foie, la croissance est extrêmement rare at apparaît seulement à 25 et 20 °C.

6. La croissance peut être obtenue sur des milieux ne contenant aucun composant homologue. Une dilution de milieu standard de 10 % environ est nécessaire pour la croissance, mais la viabilité est maintenue sur milieux non dilués.

7. On remarque l'existence d'une corrélation entre la limite thermique supérieure compatible avec le maintien des tissus d'ammocète *in vitro* et les températures les plus élevées rencontrées par l'animal entier dans son environnement naturel.

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